Investigations into ASIC desensitization

A Senior Honors Thesis

Presented in partial fulfillment of the requirements for graduation with distinction in Molecular Genetics in the undergraduate colleges of the Ohio State University

by

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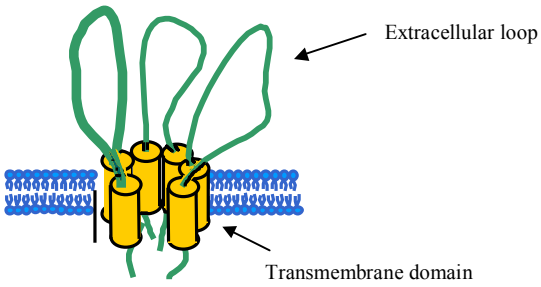
Introduction

Stroke is the third leading cause of death in the United States and survivors of stroke suffer permanent neural damage. Most strokes are ischemic, causing the blood supply to be disrupted by coagulation. Ischemia causes extracellular pH to fall. Channels known as Acid Sensing Ion Channels (ASICs) are activated under such conditions of low pH (Waldmann, 1998). When activated, ASICs are thought to be responsible for death of neuronal cell death during stroke (Xiong, et al, 2004).

ASICs are proton-gated channels that belong to the degenerin/epithelial sodium channel (Deg/ENaC) superfamily. There are six different ASIC subunits: ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4. Each ASIC subunit consists of two transmembrane domains: a large cysteine-rich extracellular motif, an intracellular carboxyl and amino terminus. ASIC subunits combine to form the quaternary functional ASIC channel as homomultimers or heteromultimers. Combinations of these subunits shape channel properties as heteromultimers (Askwith et al, 2004).



ASIC functions are not yet properly understood. In the peripheral nervous system, ASICs have been associated with mechanosensation and the perception of pain during tissue acidosis (Price et al, 2000). ASIC knock out mice do not develop chronic pain from subcutaneous injections of acidic solutions that normally evoke such a response in wildtype mice (Price, et al, 2001). In the central nervous system, ASIC1a has been demonstrated to influence plasticity, learning/memory, and fear conditioning (Wemmie et al, 2002; Wemmie et al, 2004). ASIC null mice exhibit a deficit in spatial memory and problems with eye blink conditioning. These animals also exhibit decreased long term

potentiation in the hippocampal region. Such data suggests that ASICS make significant contributions to central and peripheral nervous system functioning.

ASICs have been strongly attributed to stroke induced damage. Mice lacking ASIC1a subunits showed substantially less damage following stroke than wild-type mice (Xiong, et al, 2004). Subsequent research allowed the construction of a possible model for neuronal injury: ASICs interact with N-methyl-D-aspartate receptors (NMDAR), a type of glutamate receptor, in a cascade with Ca^{2+} activated CaMKII protein (Gao et al, 2005). Phosphorylation of ASICs at two known Ser residues enhances ASIC current and together, ASICs and NMDARs been shown to make a major contribution to ischemic neuronal injury (Gao et al, 2005).

The desensitization rate of a ligand-gated channel is the closing of a channel in the presence of an agonist. The rate of desensitization is an important property that defines the length and frequency of the response, of a channel which greatly influences the function of the channel. A fast desensitization rate causes rapid closing of the channel after activation by an agonist. Several modulating factors have been found to influence ASIC desensitization including temperature, amiloride (ASIC blocker) and a neuropeptide called FMRFamide (Phe-Met-Arg-Phe amide) (Askwith, et al, 2000; Paukert et al, 2004). Additionally, there has been a recent report that the extracellular domain in ASIC1a of fish is responsible for controlling desensitization rate (Coric, et al, 2003).

As a homomultimer, each ASIC subunit has a unique desensitization rate. ASIC1a homomultimers have a fast desensitization rate and ASIC2a homomultimers have a slow desensitization rate. To determine which domains are responsible for the different desensitization rates between ASIC1a and ASIC2a, we made chimeric subunits

containing ASIC1a and ACIC2a sequences. We analyzed their desensitization rates after expression in *Xenopus* oocytes to narrow down possible desensitization control domains.

We also used site-directed mutagenesis to identify amino acids that impact the desensitization rate of ASIC1a. By altering codons in the CNCR region (an important region for pH activation), we hoped to determine whether specific amino acid in this region can impact desensitization. Finally, after finding a relationship between pH dose response and desensitization, we analyzed the of ASIC1a and ASIC2a for possible connections between desensitization rate and pH. In essence we want to explore what makes some subunits close rapidly and allow the cell to return to normal, while others remain open dangerously long, leading to cell death.

Materials and Methods

Chimeras. The chimeras between human ASIC1a and human ASIC2a were made using the fusion PCR technique. First, PCR was done using 20 base pair ASIC1a (H1a) primers that contained 5'over-hangs of the ASIC2a (H2a) sequence and H1a template. Conversely, PCR was done with H2a primer containing 5' overhangs of the H1a sequence and H2a template. These products were mixed together for fusion PCR using H1a and H2a primers that flanked the region. This merged the 1a and 2a overhangs to form the chimera.

Site-directed mutants. Site-directed mutagenesis was done using the Quick Exchange kit from Stratagene according to manufacturer's instructions. Mutated primers were used to PCR the ASIC 1a gene inserted into a PMT3 vector. Dpn1 digest was done to eliminate methylated parental DNA. The product was then transformed into *XL1-blue* supercompetent cells for a mini-prep. The DNA samples from the mini-prep were sent

for sequencing at the Plant Genome sequencing facility at The Ohio State University for sequence verification.

Xenopus Oocyte Expression and Electrophysiology. For the measurement of whole cell currents, cDNAs of the constructs and mutants were injected into *Xenopus* oocytes. Cell recordings of oocytes were done using the two-electrode voltage clamp technique, one to four days after injection.

Data Analysis. The desensitization phase from the recordings were fit to a single exponential curve using IGOR PRO. The τ_{des} (desensitization rate) was determined from the equation $I = k_0 + k_1 \cdot e^{-t/\tau}$. I is the maximal current and t is time. A t-test was done to show the statistical significance of the desensitization rate difference between the mutants and wildtype. A p value < 0.02 was considered to be significantly different from H1a wildtype. The τ analysis was done at pH 3 for chimeras and pH 5 for the site-directed mutants.

Results

We expected chimeric channels composed of ASIC1a (H1a) and ASIC2a (H2a) to show desensitization rates that fall between ASIC1a (fast) and ASIC2a (slow). However, we found that a great majority of the chimeric channels showed a rate of desensitization (as indicated by the τ_{des}) as fast, or faster than ASIC1a homomultimeric channels. This fast desensitization rate was observed especially when an H1a sequence was present in the N-terminus in combination with an H2a C-terminus (Fig. 1*A*, *1C*).

This was unexpected since the N-terminus is located in the intracellular region and has not yet been shown to contribute to ASIC desensitization rate. This finding suggests possible interaction between the intracellular domains. Moreover, H1a presence following the “LL” region seems (located in front of the trans membrane domain) to be

adequate for an H1a like τ_{des} (Fig. 1*B*, 1*D*). This finding magnifies the importance of the intracellular and the second transmembrane domain for desensitization control.

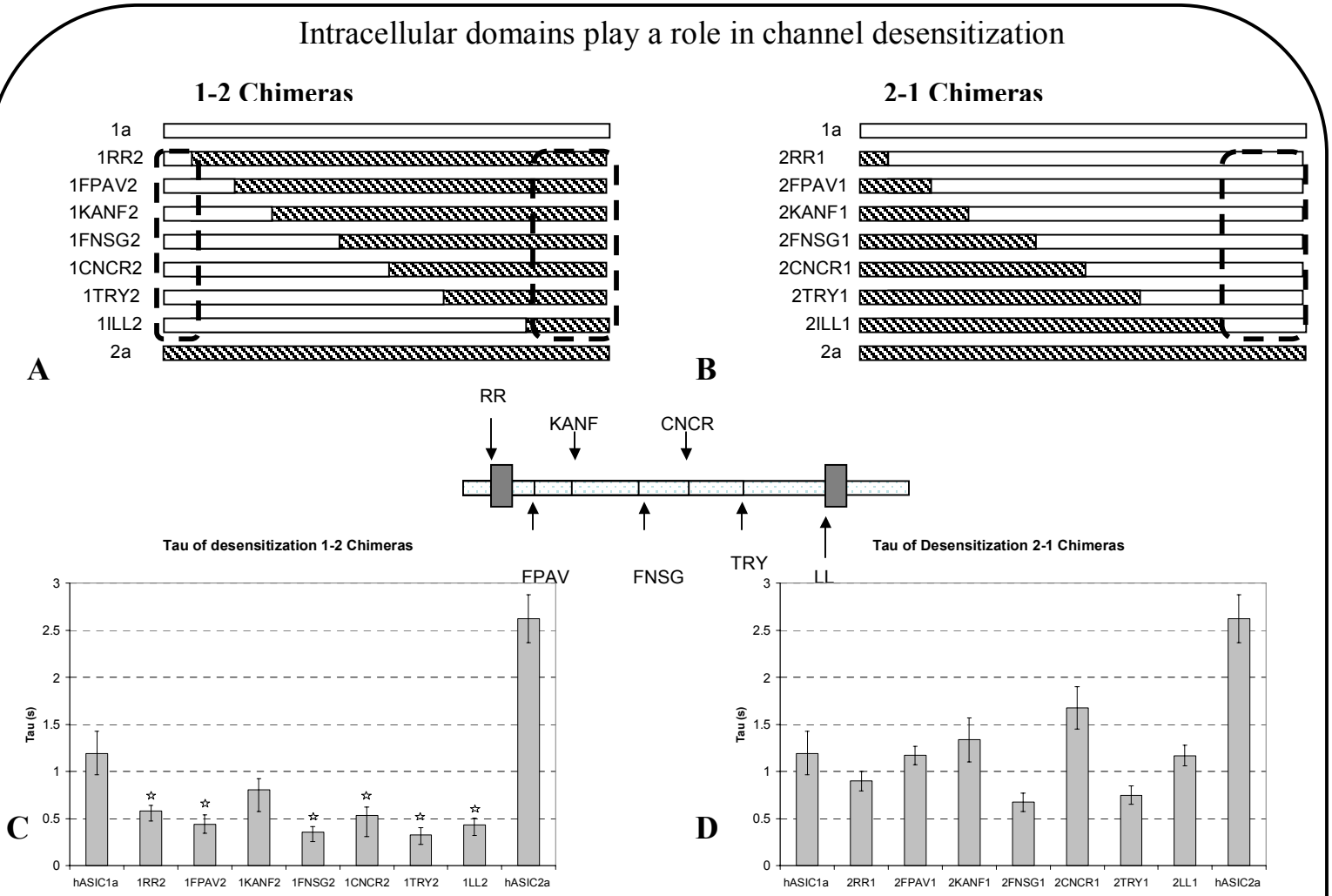


Fig. 1. (A) Schematic of chimeras with N-terminalASIC1a and C-terminal ASIC2a. The white region represents ASIC1a and the shaded region represents ASIC2a. (B) Schematic of chimeras with ASIC2a sequence N-terminal to ASIC1a . Boxes indicate regions our data suggest are important for the desensitization rate. (Middle) Diagram of ASIC protein showing localization of specific breakpoint regions. Transmembrane domains are indicated by dark rectangles. Names represent conserved amino acids of ASIC1a and ASIC2a. (C) τ_{des} of chimeras with ASIC1a sequence in the N-terminus to ASIC2a at pH3. Stars indicate statistical significance. Error bars are SEM. (D) τ_{des} of chimeras with ASIC2a sequence in the N-terminus to ASIC1a at pH of 3.

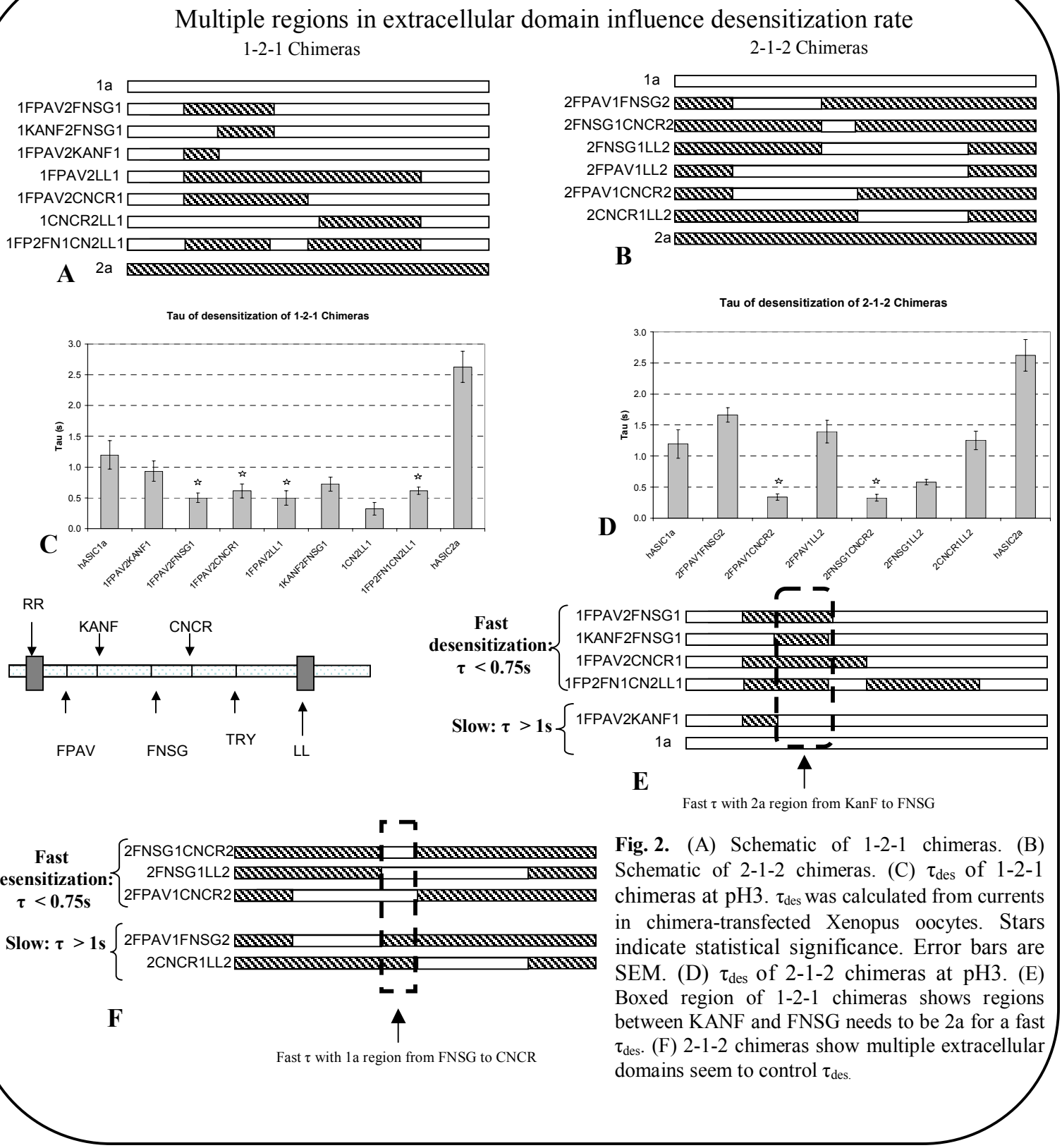
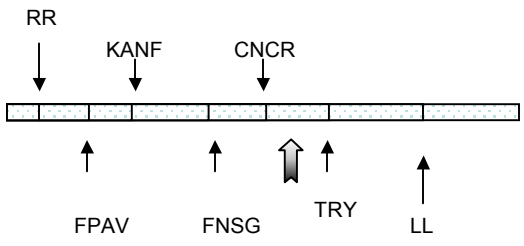


Fig. 2. (A) Schematic of 1-2-1 chimeras. (B) Schematic of 2-1-2 chimeras. (C) τ_{des} of 1-2-1 chimeras at pH3. τ_{des} was calculated from currents in chimera-transfected Xenopus oocytes. Stars indicate statistical significance. Error bars are SEM. (D) τ_{des} of 2-1-2 chimeras at pH3. (E) Boxed region of 1-2-1 chimeras shows regions between KANF and FNSG needs to be 2a for a fast τ_{des}. (F) 2-1-2 chimeras show multiple extracellular domains seem to control τ_{des}.

More complex, multifaceted chimeras with intracellular domains from either H1a or H2a were prepared to explore the impact of extracellular domain on desensitization rate (Fig. 2*A, 2B*). By analyzing chimeric channels with only specific regions of the extracellular domain changed, we hoped to further define the regions that impact desensitization. Interestingly, we again observed many chimeric channels with a rate of desensitization faster than ASIC1a or ASIC2a homomultimeric channels. This maybe due to the interaction between ASIC1a and ASIC2a protein domains. 1-2-1 chimeras with statistically significant fast desensitization (faster than ASIC1a homomultimeric channels) possess H2a sequence at the region between KANF and FNSG (Fig. 2A, 2C, 2E). We hypothesize that this ASIC2a region, in combination with an undefined ASIC1a region, causes the desensitization rate of the channel to be very fast. The 2-1-2 chimeras showed fast desensitization when there was ASIC1a presence from FNSG to CNCR (Fig. 2*B*, 2*D*, 2*F*). We hypothesize that this ASIC1a region, in combination with an undefined ASIC2a region, causes the desensitization rate of the channel to be very fast. These changes in the desensitization rates of 1-2-1 and 2-1-2 chimeras indicate that multiple regions in the extracellular domain can control channel desensitization.

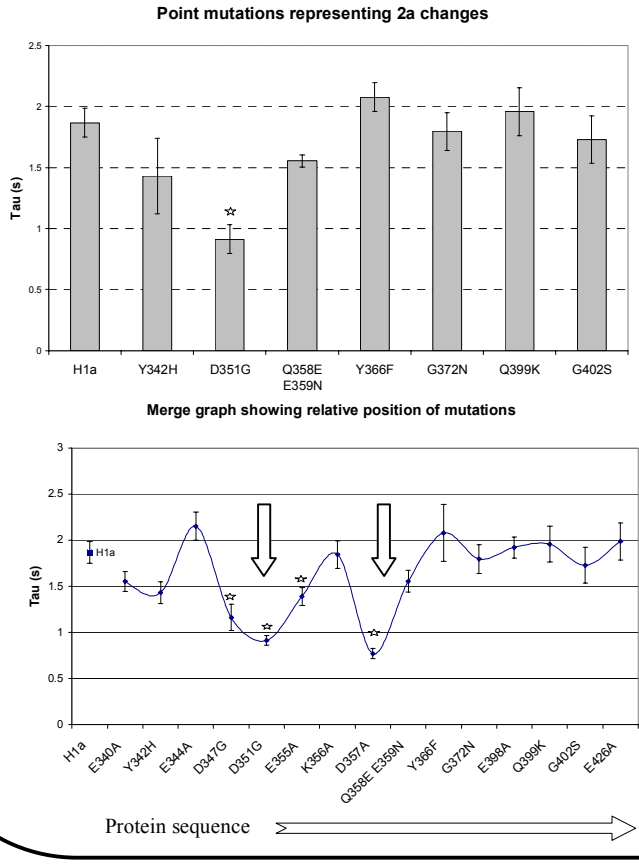
Specific amino acids influence desensitization rate. The CNCR to TRY region is a critical component to ASIC1a pH dependant activation. To determine whether specific amino acids in this region also impact desensitization, a group of site-directed mutations were made in ASIC1a. First, amino acids in this region of ASIC1a were mutated to their conserved H2a counterpart (Fig. 3*A*). The conversion of aspartic acid at 351 in ASIC1a to an uncharged glycine resulted in



a statistically significant ($p < 0.02$) 50% decrease in τ_{des}. Such a profound impact on desensitization from an H1a amino acid change to H2a at 351 suggests that this amino acid site must be essential for differentiating ASIC2a characteristics from that of ASIC1a.

The importance of charged residues for τ_{des} was investigated with point mutations that exchanged a charged amino acid such as glutamate and aspartate for an uncharged

Isolation of desensitization control regions in the CNCR region with point mutations



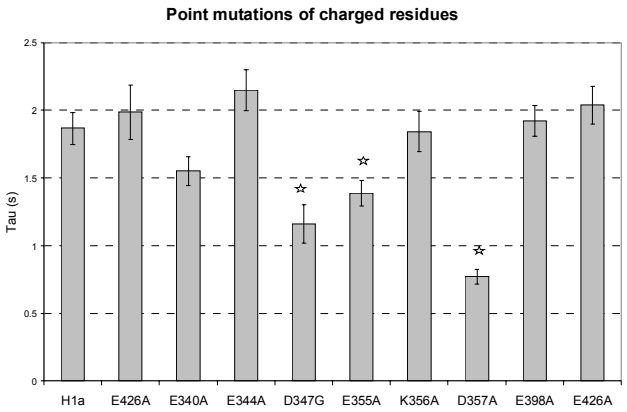


Fig. 3. Point mutations were made using the Quickchange Site-Directed Mutagenesis kit from Stratagene on H1a template. (A) Graph of site specific H1a codon change to its corresponding conserved H2a codon. (B) The τ_{des} of mutations from charged to uncharged amino acids. (C) Line graph with point mutation data merged. Mutations have been arranged left to right from N-terminus to C-terminus. All point mutation graph data was collected from ASIC activation from pH5. Statistical significance ($p < 0.02$) is indicated by ☆. Error bars are SEM.

amino acid such as alanine (Fig. 3*B*). The constructs D347G, E355A, D357A all resulted in a significantly faster τ_{des} after this modification. Their proximity to each other in the protein sequence suggests that they may be part of a domain that functions as a desensitization control center (Fig. 3*C*). In addition, adjacent amino acid alterations such as K356A, Q358E, or Q358S/E359N elicit no change (not shown); this substantiates the idea of specificity among the amino acids that influence desensitization rate.

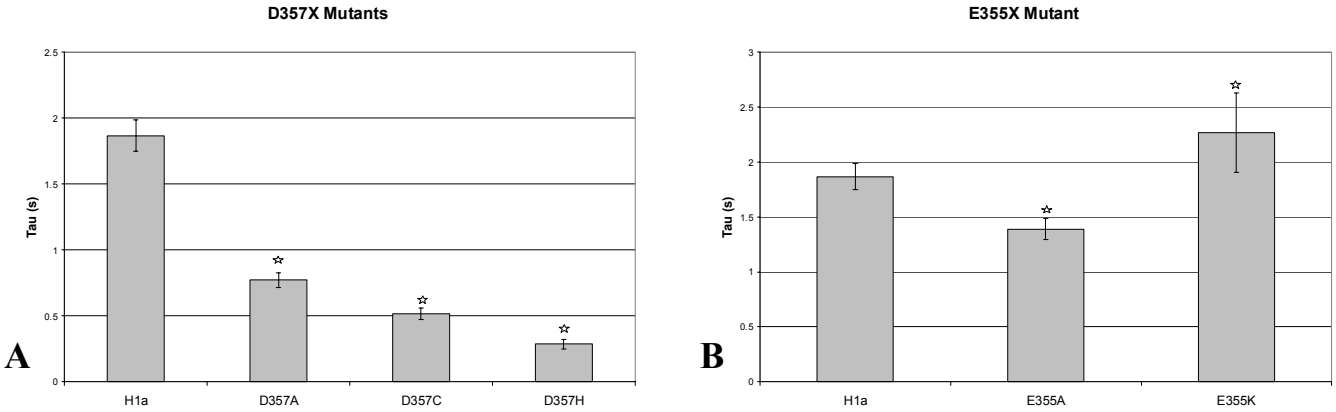


Fig. 4. (A) Graph showing desensitization of ASIC1a channels with mutations at 357 from Asp to Ala, Cys, and His (B) Graph showing desensitization of mutations at 355 from Glu to Ala, and Lys vs. Errors bars are SEM. Stars indicate statistical significance ($P < 0.02$).

To determine if the identity of substituted amino acid could impact desensitization, we changed the aspartic acid at 357 to alanine, as well as cysteine, and histidine. Each mutant yielded a progressively lower τ_{des} (Fig. 4*A*). Alanine [A] is a small amino acid that contains an uncharged methyl group. In contrast, the sulfhydryl groups of cysteine [C] (pKa = 8.6) and the immidizole group of Histidine [H] (pKA = 6.8) are weak acids: they are both protonated at physiological pH when ASICs are inactive. Carboxylic acids offer a negative charge to the protein even at a pH of 5 (pka = 5), under which pH these currents were recorded. Thus, Alanine, Cysteine and Histidine have essentially eliminated the negative charge previously provided to this protein region by Aspartate at pH 5. Therefore, losing the negative charge in this region may be the cause of faster τ_{des}. Another possible reason for the faster desensitization rate lies in the size of the amino acid side chain; the increasingly fast τ_{des} parallels an increase in size of the amino acid (Size: H>C>A). Such dependence on size can be further illustrated with the 355 mutation (Fig. 4*B*). Glutamate to Alanine alteration resulted in a statistically

significant fast $\tau_{des;}$ but the change to a bulky lysine gave a significantly slower $\tau_{des\tau.}$ The 355 and 357 mutations, together may demonstrate how steric hindrance near an ASIC desensitization control site can alter the desensitization rate.

Dose response and desensitization are linked

D351G, D357A, and E364R/M365T all experienced a decrease in pH dose response in conjunction with lower desensitization rate (data not shown). This leads us to question how pH affects desensitization rate.

Relationship between pH and τ_{des}

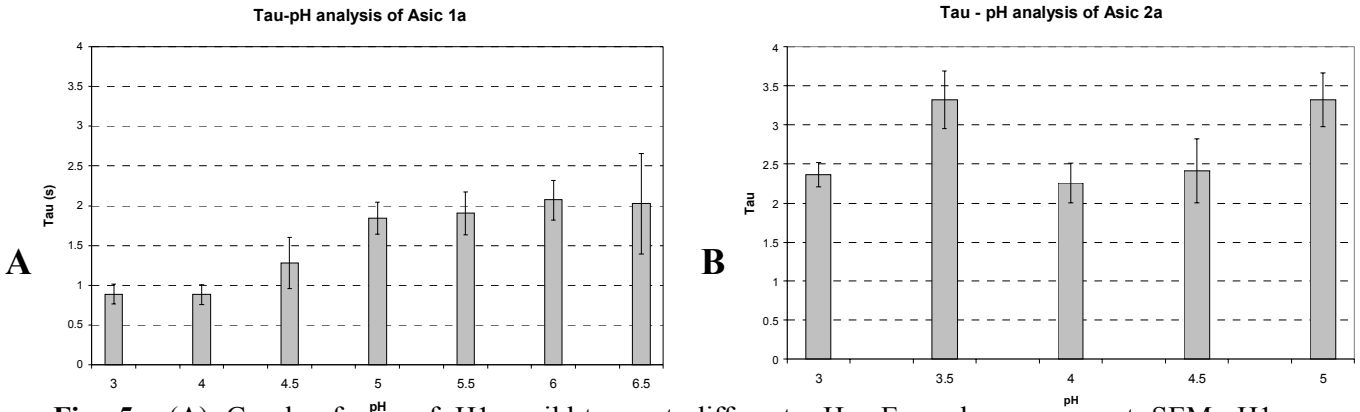


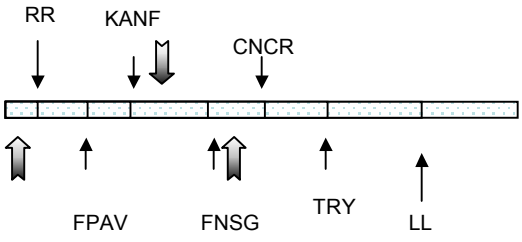
Fig. 5 (A) Graph of τ_{des}^{pH} of H1a wild-type at different pHs. Error bars represent SEM. H1a homomultimer τ_{des} at pH of various pH. The τ_{des} increases with increased pH. (B) Graph of τ_{des} of H2a wild-type at different pHs.

Human ASIC1a homomultimer τ_{des} was calculated at different pHs (Fig. 5*A*). A positive relationship was observed between τ_{des} of ASIC1a and pH - a higher pH gave slower τ_{des}. This trend was specific to ASIC1a and was not observed in the ASIC2a (Fig. 5*B*). Such a relationship suggests that an increased proton concentration facilitates rapid closing of the ASIC1a homomultimeric channel.

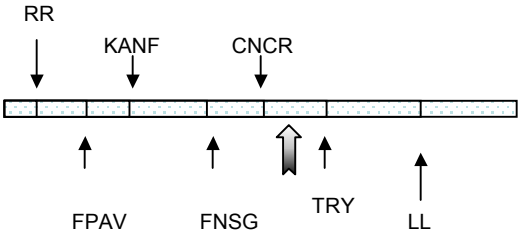
Discussion

Most chimeras and mutants showed a faster rate of desensitization than either ASIC1a or ASIC2a homomultimers. We believe that this fast rate of desensitization may mimic the fast desensitization rate of heteromultimeric channels composed of ASIC1a and ASIC2a. ASIC1a and ASIC2a co-expression yields ASIC channels with a τ_{des} faster than either ASIC1a or ASIC2a homomultimers (Askwith, et. al 2004). Similarly, ASIC1a-2a chimeras also show this trend. Although there are other explainations, it is possible that the regions we identified in our studies as generating a fast desensitization rate may represent points of contact between ASIC subunits in the multimeric ASIC channel. Further studies are necessary to test this hypothesis.

Previously, it has been shown that the ASIC1a extracellular domain in rat and fish (76% Asic 1a conservation with mammals) has significant impact on desensitization rate (Coric, et al, 2003). Our studies deduced that other regions also affect desensitization such as the N-terminus of ASIC in 1-2 chimeras, as well as the region between KANF and FNSG in 1-2-1 chimeras and FNSG to CNCR in the 2-1-2 chimeras. These areas may act as hinge regions to control the structure of ASIC, and consequently impact function. They may also be regions that destabilize the open state of the channel which causes rapid desensitization.



Point mutations made in the extracellular domain between the CNCR and TRY region revealed specific sites that have a major impact on desensitization rate. Mutations at 347, 351, 355, and 357 all had a faster desensitization rate but



desensitization rates from mutations near these regions showed minimal variation from wild type ASIC1a. This suggests that amino acids at 347-357 are essential for desensitization control and their proximity to each other maybe an indication of their interaction to carry out this function. Their interaction may form a ligand binding site or serve as important hinge regions for the open and close states of the channel

The removal of charge and modification of the size of amino acids, both had significant effects on desensitization rate. However, these effects were localized and specific to residues near amino acid 350. Altering the charge and size of the amino acid at a possible desensitization control region may affect protein interaction of the quaternary structure, and consequently modify ASIC physiology. This not only shows the importance of size and charge on desensitization but authenticates the claim that these are essential sites for desensitization control.

It has been widely accepted that there is a relationship between pH and ASIC activation but the relationship between desensitization rate and pH has never been investigated. We first observed a link between pH dose response and τ_{des} with site directed mutants. Specifically, many mutations with an altered desensitization rate also displayed a change in the pH dose response. This suggested that there maybe a relationship between pH and τ_{des}. Analysis of τ_{des} data showed a progressively increased τ_{des} with increased pH in H1a homomultimers. Such a relationship alludes to some type of proton modification of ASIC1a homomultimer either directly or indirectly by a pathway.

The intracellular domain of human ASIC is now a new target for research in search of desensitization control mechanisms. We have also established a relationship between pH and desensitization rate. In effect this data puts forth a strong connection

between the concentration of protons and ASIC1a desensitization which may direct future studies to specific pathways that may regulate this interaction. Finally, we have narrowed down some very promising amino acids in the extracellular region that have major effects on desensitization. Continued research on the mechanics of desensitization rate may eventually be of help to stroke patients. The discovery of domains responsible for ASIC desensitization can potentially lead to development of drugs that can target and alter ASIC desensitization rate during stroke to minimize neuronal damage.

References

Askwith, C.C., Cheng, C., Ikuma, M., Benson, C., Price, M.P., and Welsh, M.J. (2000). Neuropeptide FF and FMRFamide potentiate acid-evoked currents from sensory neurons and proton-gated DEG/ENaC channels. Neuron *1,* 133-141.

Askwith, C.C., Wemmie, J.A., Price, M.P., Rokhlina, T., and Welsh, M.J. (2004). Acid-sensing ion channel 2 (ASIC2) modulates ASIC1 H+-activated currents in hippocampal neurons. J. Biol. Chem. *18,* 18296-18305.

Coric, T., Zhang, P., Todorovic, N., and Canessa, C.M. (2003). The extracellular domain determines the kinetics of desensitization in acid-sensitive ion channel 1. J. Biol. Chem. *46,* 45240-45247.

Gao, J., Duan, B., Wang, D.G., Deng, X.H., Zhang, G.Y., Xu, L., and Xu, T.L. (2005). Coupling between NMDA receptor and acid-sensing ion channel contributes to ischemic neuronal death. Neuron *4,* 635-646.

Paukert, M., Babini, E., Pusch, M., and Grunder, S. (2004). Identification of the Ca2+ blocking site of acid-sensing ion channel (ASIC) 1: implications for channel gating. J. Gen. Physiol. *4,* 383-394.

Price, M.P., Lewin, G.R., McIlwrath, S.L., Cheng, C., Xie, J., Heppenstall, P.A., Stucky, C.L., Mannsfeldt, A.G., Brennan, T.J., and Drummond, H.A. *et al*. (2000). The mammalian sodium channel BNC1 is required for normal touch sensation. Nature *6807,* 1007-1011.

Price, M.P., McIlwrath, S.L., Xie, J., Cheng, C., Qiao, J., Tarr, D.E., Sluka, K.A., Brennan, T.J., Lewin, G.R., and Welsh, M.J. (2001). The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice. Neuron *6,* 1071-1083.

Waldmann, R., and Lazdunski, M. (1998). H(+)-gated cation channels: neuronal acid sensors in the NaC/DEG family of ion channels. Curr. Opin. Neurobiol. *3,* 418-424.

Wemmie, J.A., Chen, J., Askwith, C.C., Hruska-Hageman, A.M., Price, M.P., Nolan, B.C., Yoder, P.G., Lamani, E., Hoshi, T., Freeman, J.H.,Jr, and Welsh, M.J. (2002). The acid-activated ion channel ASIC contributes to synaptic plasticity, learning, and memory. Neuron *3,* 463-477.

Wemmie, J.A., Coryell, M.W., Askwith, C.C., Lamani, E., Leonard, A.S., Sigmund, C.D., and Welsh, M.J. (2004). Overexpression of acid-sensing ion channel 1a in transgenic mice increases acquired fear-related behavior. Proc. Natl. Acad. Sci. U. S. A. *10,* 3621-3626.

Xiong, Z.G., Zhu, X.M., Chu, X.P., Minami, M., Hey, J., Wei, W.L., MacDonald, J.F., Wemmie, J.A., Price, M.P., Welsh, M.J., and Simon, R.P. (2004). Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels. Cell *6,* 687-698.